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Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 March 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

While the referees appreciate the data supporting that OTUB1 regulates the stability of p53, they also raise major concerns with the analysis. As you can see below, all three referees find that there is not enough data provided to support the mechanism for how OTUB1 regulates the stability of p53 and if OTUB1 acts in a canonical manner or non-canonical manner to regulate p53 levels. This is one the major concerns with the study and this issue has to be resolved before further consideration here. Should you be able to provide further data to provide definitive insight into the mechanism whereby OTUB1 regulates p53 stability and the other concerns raised then we would be willing to look at a revised manuscript. I don't know if you will be able to address the concerns raised in full, but it is such level of insight that we would need in order to consider publication in the EMBO Journal. I should remind you that it is EMBO Journal policy to allow a single major round of revision and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I also realize that addressing all of the referees' criticisms might require a lot of additional time and effort and be technically challenging, and I would also understand it if you were to rather decide to publish the manuscript rapidly and without any significant changes elsewhere. If you decide to thoroughly revise the manuscript and submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our

Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The manuscript by Sun and Dai presents evidence that the deubiquitylating enzyme OTUB1 stabilizes p53 and enhances expression of two of its responsive genes, MDM2 and p21. Ubiquitylation is a major regulatory mechanism of 53 stability and in addition to MDM2, numerous other ubiquitin ligases have been proposed to be involved as well as deubiquitylating enzymes USP2, USP7, and USP10. The present work has identified another deubiquitylating enzyme, OTUB1, whose over-expression stabilizes p53. The authors propose OTUB1 counteracts p53 ubiquitylation by MDM2 and that this may be mediated by a direct interaction between OTUB1 and p53. Although the overall technical quality of the data appears high, it is surprising and disappointing that the authors did not explore an alternative mechanism. The recent work by Nakada et al. demonstrates that OTUB1 binds directly to UBC13 and inhibits its ability to generate K63-linked ubiquitin chains through a mechanism that does not rely on its catalytic cysteine. These authors also determined that the E2s proposed to function with MDM2 to promote p53 ubiquitylation--UbcH5a/b/c--also bind to OTUB1. In light of this compelling model, one is left unsatisfied with the present work as the experimental data could be explained by this mechanism rather than through p53 binding. If the authors intend to propose that p53 is regulated by both canonical and non-canonical mechanisms involving this deubiquitylating enzyme, it is reasonable to expect evidence presented to support these claims.

Specific Points

1. The authors need to explain better their rationale for focusing on OTUB1, as it is perplexing how they would serendipitously find that p53 is stabilized by OTUB1.

2. Both MDM2 and p21 are known to be regulated post-translationally through the ubiquitin proteasome system. The authors need to show half-life measurements for these proteins as well as include p53 transcript abundance measurements.

3. It is unclear how many times the half-life measurements in Figure 1F were performed as error bars are absent.

4. For NiNTA pulldown experiments (e.g., Figure 3A, 5C, 6D), the authors need to explain why unmodified p53 is recovered.

5. Although the authors do a nice job in showing the input material in Figure 4B, similar input westerns on the same blot are necessary to judge the strength of interaction shown in Figure 4A.

6. The experiments in Figure 7 examining a proposed dominant negative function of D88A need to be done with wild-type OTUB1 to allow for comparison.

7. The claim that p53 unable to be ubiquitylated could not be deubiquitylated and thereby not released from OTUB1 needs to be substantiated and clarified.

Referee #2:

Otubain 1 (Otub1) is a previously characterized deubiquitinating enzyme. In this manuscript, the authors present data to argue that Otub1 regulates p53 activity after DNA damage by antagonizing the effects of the ubiquitin ligase for p53, Mdm2. The findings are carefully presented and a reasonable case is made for the relevance of Otub1 in positive regulation of p53. However, there are several important concerns about the proposed mechanism of action that need to be addressed before the study is suitable for publication in EMBO J.

1. Most notably, the authors present a model for the effects of Otub1 as being mediated by a direct effect on p53 itself. Previously another deubiqutinating enzyme HAUSP was proposed to do have a similar effect on p53, but then was subsequently shown to be acting via effects on Mdm2. The increase in Mdm2 levels that is seen after upregulation of Otub1 could also be explained by Otub1 deubiquitinating Mdm2. The authors need to explore this possibility. It is also unclear whether the Mdm2 protein after increases in Otub1 expression remains functional as a ubiquitin ligase. It is also possible that Otub1. The need for a direct interaction between Otub1 and p53 is shown for in vitro ubiquitination. But given the results with the C91 mutants it is unclear how relevant the in vitro assay is to what happens in cells.
2. The evidence for apoptosis is based on measurement of hypodiploid DNA content. For the experiment in Figure 2C, other more definitive assays are needed such as annexin V staining or TUNEL.
3. The p53-dependence of the effects on cellular outcome by Otub1 is a critically important element of the study. This needs to be shown using isogenically matched cells that only differ in p53 status. U2OS and Saos-2 are tumor cell lines that differ in multiple ways besides just p53 status.
4. The colony assays that are shown in the manuscript should be quantitated and the results of multiple experiments shown as a graph with statistical analysis provided (for Figures 2D and 6E).
5. The effect of the C91 mutants on p53 in spite of showing that they are not capable of deubiquitinating p53 in vitro is troubling. The authors need to provide a more mechanistic explanation for this outcome as it raises questions about whether Otub1 is indeed acting on p53 as a deubiquitinating enzyme.
6. The authors hypothesize that the D88A mutant acts in a dominant-negative manner. This needs to be confirmed by showing that it has no additional effect in cells that have been depleted of Otub1 by RNAi for example.

Referee #3:

This manuscript reports the identification of a new deubiquitylating enzyme, Otub1, active towards p53.

The authors demonstrate that the overexpression of this DUB but not of its homolog , Otub2, is able to deubiquitinate and stabilize p53. This results in the transcriptional activation of p53-targets p21, Mdm2 and Bax, increase of cells in sub-G1 phase and inhibition of U2OS cells colony forming ability.

The authors further map the p53 and Otub1 domains responsible for their binding and find that these overlap the DNA binding domain in p53 and the amino and carboxyl termini in Otub1.
They demonstrate that the canonical catalytic site Cys91 of Otub1 is not required for p53 stabilization whereas it is the residue Asp88. They further show that the mutant Otub1D88A behaves as a dominant negative protein able to reduce protein levels of p53 and of its target genes, p21, Mdm2 and Bax. However, this does not result in an increase of colony forming ability.
Finally, the authors indicate that Otub1 is a critical factor for p53 activation in response to DNA damage. This is deduced by the observations that: 1) the overexpression of the dominant negative Otub1S188A reduces p53 protein levels induction in response to DNA damage by NCS, Eto, and UVC; 2) the knockdown of endogenous Otub1 in U2OS cells reduces p53 protein levels following Etoposide, NCS, 5-Fluorouracyl and UVC; 3) the binding between Otub1 and p53 increases upon Etoposide treatment.

The authors propose a model to reconcile their data with those existing in literature hypothesizing that Otub1 regulates cytoplasmic p53 levels whereas the other p53-DUBs, USP7 and USP10, regulate p53 in the nucleus.

The experiments presented are well done, the results very clear. The activity of Otub1 towards p53 stabilization is convincing.

I think two main points are not entirely solved in this work:

1) The molecular mechanism by which Otub1 functions. Otub1 is a particular deubiquitylating enzyme. It may act in a canonical way by detaching bound ubiquitin or in a non-canonical way by blocking the E2/E3 enzyme preventing the attachment of ubiquitin molecules to the target.

The authors suggest that their results support the canonical way although in the Discussion (pg 20) they hypothesize that both pathways (canonical and non-canonical) may function in p53 stabilization. I think they provide no proofs for the canonical one whereas various evidences suggest that Otub1 may function by interfering with ubiquitin ligase Mdm2:
• the catalytically inactive Otub1-C91A mutant is still effective in stabilizing p53;
• the inactive Otub1-D88A mutant is inactive in deubiquitinating p53 in vitro but is effective in H1299 cells;
• Exogenous Mdm2 is stabilized as well as or even more of p53 by Otub1 and reduced by Otub1D88A mutant (Fig. 1D, 5B, 6C);
• In the Boston-Bachem in vitro ubiquitination kit, it is present Mdm2. So the efficacy of wt-Otub1 in this assay does not exclude this hypothesis.
The authors should definitely ascertain the mechanism, especially in order to understand the role of Otub1 in human cancers (Discussion pg21). They should demonstrate the direct binding between p53 and Otub1 and the role of Mdm2 in it. They could use other p53 E3 ligases in order to verify the function of Otub1. They should ascertain whether the dominant negative function of mutant D88A is due to substrate-trapping or to an increased degradative activity of Mdm2 (as suggested by Figure 6C).

2) The effectiveness of Otub1 in the p53-mediated DNA damage response. Otub1 has been describes as an inhibitor of DNA repair. So the hypothesis that Otub1 functions in promoting DNA damage response is somehow contradictory and should be firmly demonstrated.
• The authors should show some cellular data to verify the cell response upon expression of Otub1-D88A mutant or Otub1 siRNA (cell cycle, apoptotic assays, colony formation etc.). Furthermore, a time course analysis of Otub1 levels and of the other proteins would be more convincing. In addition, analysis of p53 activity should be verified (at least by RT-PCR of its target genes) under these conditions.
• The cytoplasmic localization of Otub1 should be confirmed in other cell lines and especially in non-transformed cells; the p53-Otub1 binding, in a time course analysis, should be verified in the cytoplasmic compartment to confirm the model the authors suggest. Moreover, p53 unable to localize at the cytoplasm (by mutation of NES or leptomycin B treatment) should be used to confirm the model.
• Finally, the results in figure 7 and 8 evidence a different activity of Otub1 following UVC in comparison to the other DNA damaging agents. Indeed, Otub1 seems ineffective in altering p53 levels upon UVC. This could be related to the dose of UVC used: 40J. This is probably a lethal dose that activates the apoptotic response (In fact, p21 levels decrease). This could discriminate Otub1 function following different cell responses rather than in different cell compartments. This could also solve the contradiction of Otub1 function in DNA damage response. As it has been suggested, Otub1 may set the threshold for initiating a transient DNA damage response (resulting in a growth arrest) (Nakada 2010). Conversely, its inactivation might switch on a permanent and fatal cell death response. The authors should verify and compare the functionality of Otub1 between these cell responses.

Specific points:
1. Fig.1B: in the results (pg. 7) the authors mention p21. However, the blot of p21 is missing.
2. Figure 2B: FACS analysis is not reliable as apoptotic assay (Kroemer et al., Cell Death and Diff. 2009) and the signal of PARP cleavage is really weak. Authors should perform more specific assays (TUNEL, annexin, caspase-3 activation etc.)

• MDMX is also known as MDM4. For clarity, the authors should report the double nomenclature at the beginning.
• In Fig 4B, specificity of the binding between endogenous proteins should be confirmed by siRNA against p53 or Otub1.
• The authors should report the cell line used in each panel of the figures. This would increase reading clarity.

1st Revision - authors' response 16 September 2011

Responses to reviewers' comments:

Response to Reviewer #1:

"*Although the overall technical quality of the data appears high, it is surprising and disappointing that the authors did not explore an alternative mechanism. The recent work by Nakada et demonstrates that…. In light of this compelling model, one is left unsatisfied with the present work as the experimental data could be explained by this mechanism rather than through p53 binding".*
Our response: The reviewer is correct and it is indeed important to demonstrate the non-canonical mechanism, as the catalytic activity is not required for Otub1 to regulate p53 in cells. This critical point was also raised by the reviewers #2 and #3.
We have now shown (1). Wild-type Otub1 and Otub1C91S, but not Otub1D88A, markedly inhibited MDM2-mediated p53 ubiquitination *in vitro* (new Figs 7A and 7B); (2). Wild-type Otub1 and Otub1C91S, but not Otub1D88A, directly suppresses the Ubconjugating activity of UbcH5 *in vitro* (New Figs. 7C and 7D and supplementary Fig. S7A) in the absence or presence of MDM2; (3) Wild-type Otub1 and Otub1C91S, but not Otub1D88A, interacts with UbcH5 (New Fig. 7E, 7F and new supplementary Fig. S7CS7D). These results clearly demonstrate that Otub1 directly suppresses p53 ubiquitination via non-canonical inhibition of UbcH5, although we cannot exclude the possibility that Otub1 also suppresses MDM2 E3 activity. This mechanism has been suggested by Nakada et al (2010) showing that Otub1 suppresses DNA damage-dependent chromatin ubiquitination via suppression of Ubc13.
We also agree with the reviewer #3 that our previous *in vitro* deubiquitination assay system using *in vitro* ubiquitinated p53 contains UbcH5 and MDM2, which might affect our interpretation. Therefore, we performed the assay using ubiquitinated p53 purified from cells. Our new result also showed that Otub1 slightly reduced the polyubiquitinated p53, resulting in a slight increase of monoubiquitinated p53 (New supplementary Fig. S5B), suggesting that Otub1 possesses weak DUB activity towards polyubiquitinated p53. Given that Otub1 directly interacts with p53 (New Fig. 3C), we believe that Otub1 regulates p53 ubiquitination via both mechanisms. Based on these data, we have modified our model of Otub1 regulation of p53 in new Fig. 10F.

Specific Points:
1. "The authors need to explain better their rationale for focusing on OTUB1, as it is perplexing how they would serendipitously find that p53 is stabilized by OTUB1."
Our response: Our initial goal was to identify deubiquitinating enzymes (DUBs) other than USP family members that can regulate p53. We started to explore the second largest DUB family, the OTU family DUBs. We first cloned several OTU DUBs, including Otub1 and Otub2. Our initial test luckily showed that Otub1, but not Otub2, stabilized p53. Therefore, we focused on Otub1 instead of further screening the whole OTU family. We have incorporated this into the revised text.

2. "Both MDM2 and p21 are known to be regulated post-translationally through the ubiquitin proteasome system. The authors need to show half-life measurements for these proteins as well as include p53 transcript abundance measurements."
Our response: We have now measured the half-life of MDM2 and p21 and found that Otub1 prolonged the half-life of MDM2, but not p21 (new supplementary Figs S1F and S1G). This is consistent with our finding that Otub1 stabilizes MDM2 and suppresses

MDM2 autoubiquitination (new supplementary Fig. S6E). RT-qPCR assays showed that Otub1 does not increase the p53 mRNA levels (new Fig S1E), consistent with the notion that Otub1-mediated regulation of p53 occurs at the posttranslational level.

3. "It is unclear how many times the half-life measurements in Figure 1F were performed as error bars are absent."
Our response: We have performed the p53 half-life measurements three times and the error bars are included in the new Fig. 1F.

4. "For Ni-NTA pulldown experiments (e.g., Figure 3A, 5C, 6D), the authors need to explain why unmodified p53 is recovered".
Our response: The non-specific pulldown of a portion of unmodified p53 is common in the *in vivo* ubiquitination assays using Ni2+-NTA pulldown (eg. Stad, et al, EMBO Report, 2001; Xiong, et al, Oncogene, 2011; Sun, et al, JBC, 2007, etc.). The reason for this is not known. One possibility is that there are several His stretches in the p53 protein that may fold to form a binding site to Ni2+.

5. " Although the authors do a nice job in showing the input material in Figure 4B, similar input westerns on the same blot are necessary to judge the strength of interaction shown in Figure 4A".
Our response: This has been done as suggested (*new Fig. 4A*).

6. "The experiments in Figure 7 examining a proposed dominant negative function of D88A need to be done with wild-type OTUB1 to allow for comparison".
Our response: This has been done as suggested (*new Fig. 8D*).

7. "The claim that p53 unable to be ubiquitylated could not be deubiquitylated and thereby not released from OTUB1 needs to be substantiated and clarified".
Our response: This is a prediction based on the substrate-trapping mechanism that has been known for protein tyrosine phosphatase (PTP) mutants. Such mutants would cause blocking of ongoing PTP catalysis, leading to the trapping of the substrate in the catalytic pocket of the PTP and stabilization of the enzyme-substrate interaction. As a number of studies have emphasized the role of the C-terminal lysine residues (370, 372, 373, 381, 382, and 386) in MDM2-mediated p53 ubiquitination and degradation (Rodrigues MS, et al, 2000; Nakamura S, et al, 2000, 2002). Mutation of all the six lysines renders p53 resistant to MDM2-mediated p53 ubiquitination and proteasome degradation. Thus we hypothesized that our observed strong binding of Otub1 to the Cterminal deleted p53 mutant might be due to a similar substrate-trapping mechanism, as these non-ubiquitinated p53, once binding to Otub1, would form a stable complex as no enzymatic reaction will be catalyzed, considering that Otub1 has weak DUB activity towards p53. The same might be true for the strong binding of the D88A mutant to p53 (Fig 6E).

Response to Reviewer #2:

"The findings are carefully presented and a reasonable case is made for the relevance of Otub1 in positive regulation of p53. However, there are several important concerns about the proposed mechanism of action that need to be addressed before the study is suitable for publication in EMBO J."

1. "Most notably, the authors present a model for the effects of Otub1 as being mediated by a direct effect on p53 itself. Previously another deubiqutinating enzyme HAUSP was proposed to do have a similar effect on p53, but then was subsequently shown to be acting via effects on Mdm2. The increase in Mdm2 levels that is seen after upregulation of Otub1 could also be explained by Otub1 deubiquitinating Mdm2. The authors need to explore this possibility. It is also unclear whether the Mdm2 protein after increases in Otub1 expression remains functional as an ubiquitin ligase. The reviewer also asked whether Otub1 directly interacts p53 in vitro (It is also possible that Otub1. The need for a direct interaction between Otub1 and p53 is shown for in vitro ubiquitination). But

given the results with theC91 mutants it is unclear how relevant the in vitro assay is to what happens in cells".
Our response: These are important questions. The reviewer is correct. Otub1 also interacts with MDM2 and suppresses its autoubiquitination, leading to its stabilization in cells (New supplementary Figs. S7, 1F and 1G). However, our new data suggest that Otub1 primarily suppress UbcH5 (E2), thus interfering with MDM2 E3 activity and leading to suppression of MDM2-mediated p53 ubiquitination (new Figs 7A to 7D). In this case, the stabilized MDM2 would not be functional as the E2 is suppressed by Otub1. This is different from the case of HUASP. HAUSP preferentially deubiquitinates MDM2. Thus substantial knockdown of HUASP destabilizes MDM2, leading to p53 stabilization. Substantial knockdown of Otub1 does not stabilize p53 (Fig. 9).
The Otub1-p53 interaction is direct as both proteins purified from bacteria bind to each other *in vitro* (New Fig. 3C). Otub1 also possesses weak DUB activity towards polyubiquitinated p53 (New Fig. S5B). We agree at this point with the reviewer regarding whether this DUB activity plays a significant function in regulating p53 in cells, considering that C91S still suppresses MDM2-mediated p53 ubiquitination in cells and *in vitro*. However, it does not discount our finding that Otub1 plays a critical role in regulating p53 ubiquitination and stability.

2. "The evidence for apoptosis is based on measurement of hypodiploid DNA content. For the experiment in Figure 2C, other more definitive assays are needed such as annexin V staining or TUNEL".
Our response: Thank you for the suggestion. We have now performed Annexin V staining and found that overexpression of Otub1 significantly induced the percentage of Annexin V-positive cells (New Fig. 2B). Altogether, we confirmed that overexpression of Otub1 induces apoptosis using multiple lines of evidence (PI staining, Annexin V staining, and PARP cleavage).

3. "The p53-dependence of the effects on cellular outcome by Otub1 is a critically important element of the study. This needs to be shown using isogenically matched cells that only differ in p53 status. U2OS and Saos-2 are tumor cell lines that differ in multiple ways besides just p53 status".
Our response: We agree with this reviewer and performed colony assays using HCT116p53+/+ and HCT116p53-/- isogenic cell lines. As shown in the new supplementary Figs. S2G and 2H, induced expression of Otub1 greatly suppressed colony formation in HCT116p53+/+, but not HCT116p53-/- cells. These results clearly support that Otub1 suppresses cell growth by inducing p53-dependent apoptosis.

4. "The colony assays that are shown in the manuscript should be quantitated and the results of multiple experiments shown as a graph with statistical analysis provided".
Our response: We have now included the quantitative data from three independent experiments in new supplementary Fig. S2E. Also, the quantitative results from HCT116p53+/+ and HCT116p53-/- isogenic cell lines were shown in new Fig. S2G.

5. "The effect of the C91 mutants on p53 in spite of showing that they are not capable of deubiquitinating p53 in vitro is troubling. The authors need to provide a more mechanistic explanation for this outcome as it raises questions about whether Otub1 is indeed acting on p53 as a deubiquitinating enzyme".
Our response: This is indeed a very important question. We have now performed *in vitro* deubiquitination assays using ubiquitinated p53 purified from cells and demonstrate that Otub1 possesses weak DUB activity towards polyubiquitinated p53 (Fig. S5B). We have also characterized the non-canonical mechanism for Otub1 regulation of p53 as discussed in our response to the overall comment by the reviewer 1#.

6. "The authors hypothesize that the D88A mutant acts in a dominant-negative manner. This needs to be confirmed by showing that it has no additional effect in cells that have been depleted of Otub1 by RNAi for example".
Our response: Thanks for the suggestion. We have now shown that depletion of Otub1 did not further reduce the effect of D88A mutant on p53 levels (New supplementary Fig. S4B), suggesting that the D88A mutant most likely acts as a dominant-negative mutant.

This might be due to that the strong interaction of D88A with p53 mutant would interfere with the interaction of endogenous Otub1 with p53.

Response to Reviewer #3:

"The experiments presented are well done, the results very clear. The activity of Otub1 towards p53 stabilization is convincing. I think two main points are not entirely solved in this work"

1. "The molecular mechanism by which Otub1 functions. Otub1 is a particular deubiquitylating enzyme. It may act in a canonical way by detaching bound ubiquitin or in a non-canonical way by blocking the E2/E3 enzyme preventing the attachment of ubiquitin molecules to the target. The authors suggest that their results support the canonical way although in the Discussion (pg 20) they hypothesize that both pathways (canonical and non-canonical) may function in p53 stabilization. I think they provide no proofs for the canonical one whereas various evidences suggest that Otub1 may function by interfering with ubiquitin ligase MDM2. (1) the catalytically inactive Otub1-C91A mutant is still effective in stabilizing p53; (2) the inactive Otub1-D88A mutant is inactive in deubiquitinating p53 in vitro but is effective in H1299 cells; (3) Exogenous Mdm2 is stabilized as well as or even more of p53 by Otub1 and reduced by Otub1D88A mutant (Fig. 1D, 5B, 6C); (4) In the Boston-Bachem in vitro ubiquitination kit, it is present Mdm2. So the efficacy of wt-Otub1 in this assay does not exclude this hypothesis. The authors should definitely ascertain the mechanism, especially in order to understand the role of Otub1 in human cancers. They should demonstrate the direct binding between p53 and Otub1 and the role of Mdm2 in it. They could use other p53 E3 ligases in order to verify the function of Otub1. They should ascertain whether the dominant negative function of mutant D88A is due to substrate-trapping or to an increased degradative activity of Mdm2 (as suggested by Figure 6C)."
Our response: These are excellent questions. The major point here is the molecular mechanism underlying Otub1 regulation of p53 ubiquitination, which was raised by all the reviewers. First, we agree with this reviewer that the *in vitro* assay of old Fig. 4D contains MDM2 and E2 and thus does not exclude the possibility that Otub1 still acts on E2/E3. We have now performed the *in vitro* deubiquitination assay using ubiquitinated p53 purified from cells. Our new result showed that Otub1 has weak deubiquitinating activity towards polyubiquitinated p53 *in vitro*, which requires the catalytic C91 residue (Fig. S5B). However, as discussed in our response to the overall comment by the reviewer 1#, we have now shown that Otub1 suppress the activity of UbcH5 (E2), leading to suppression of MDM2-mediated p53 ubiquitination (a non-canonical mechanism as proposed for Otub1 regulation of Ubc13). As to whether Otub1 suppresses E3, our results do not exclude this possibility, considering Otub1 also binds to MDM2. It is most likely that Otub1 suppresses p53 ubiquitination by interfering the whole E2-E3 complex. Actually DUBs often associate with E3s (Nijman S, et al. 2005)

In addition, we confirmed that Otub1 interacts with both p53 and MDM2 directly *in vitro* using GST-pull down assays (New Figs. 3C and new supplementary Figs. S6B, S6C). Also, Otub1 forms a complex with MDM2 and p53 in cells (new supplementary Fig. S6D). We examined whether Otub1 affect p53 ubiquitination by ARF-BP1, which also use UbcH5 as an E2, and found that Otub1 also suppresses ARF-BP1-mediated p53 ubiquitination in cells (new supplementary Fig. S3D). We think that the dominant negative function of the D88A mutant on p53 is likely due to substrate-trapping, but not the activity of MDM2, as MDM2 is reduced by overexpression of D88A (Fig. 6C). This would presumably lead to stabilization of p53. Because D88A binds to p53 much stronger than wild-type Otub1 (Fig. 6F), it may interfere with the binding of endogenous Otub1 to p53. Supporting this, we have shown that depletion of Otub1 did not further reduce the effect of D88A mutant on p53 levels (New supplementary Fig. S4B). Overall, our data support that Otub1 regulates p53 via a non-canonical mode in cells.

2. "The effectiveness of Otub1 in the p53-mediated DNA damage response. Otub1 has been describes as an inhibitor of DNA repair. So the hypothesis that Otub1 functions in

promoting DNA damage response is somehow contradictory and should be firmly demonstrated".
Our response: It has been shown recently by Nakada et al that overexpression of Otub1 inhibits DSB-induced chromatin ubiquitination by antagonizing Ubc13 (E2), leading to inhibition of DNA repair. Consistently, knockdown of Otub1 mitigates the DSB repair defects associated with defective ATM signaling (*Nature 466: 941, 2010*). To our point of view, our finding is functionally consistent with this study. Assumingly, inhibition of DNA damage repair by Otub1 overexpression would result in sustained DSB, which would cause sustained p53 activation. Otub1-mediated suppression of p53 ubiquitination also contributes to p53 stabilization and activation. So it is likely that Otub1 could have a dual function in activating p53 signaling: suppression of DSB repair and MDM2-mediated p53 ubiquitination following DNA damage, leading to apoptosis. This may occur when cells receive lethal DNA damaging signal and need to be eliminated from the proliferation pool.
However, I also agree with the reviewer's notion about Otub1 in setting threshold for DNA damage response (see below comments 3). Under physiological conditions, endogenous Otub1 may set a threshold for RNF168 to control chromatin ubiquitination. Upon DNA damage, Otub1 may dissociate from Ubc13-RNF168 complex allowing RNF168 to catalyze ubiquitination of chromatin at DNA damage sites (Nakada et al, 2010), whereas it associates with the UbcH5-MDM2 complex to suppress MDM2-mediated p53 ubiquitination. This dual action would likely work in concert to promote DNA damage response by initiating cell cycle arrest and subsequent DNA repair. It is certain important in future studies to examine the connection between Otub1's role in the p53 pathway and the mode proposed by Nakada et al. (2010). However, we believe that we have provided strong data supporting the role of Otub1 in regulating p53 response to DNA damage.

(1) The authors should show some cellular data to verify the cell response upon expression of Otub1-D88A mutant or Otub1 siRNA (cell cycle, apoptotic assays, colony formation etc.). Furthermore, a time course analysis of Otub1 levels and of the other proteins would be more convincing. In addition, analysis of p53 activity should be verified (at least by RT-PCR of its target genes) under these conditions".
Our response: We have now included experiments examining the cellular response upon Otub1 knockdown. We showed that knockdown of Otub1 significantly attenuated DNA damage-induced G2/M and S phase checkpoints (New Figs. 9I and 9J). As we have not been successful in establishing tet-inducible Otub1 shRNA cell line, it is not feasible at this time for us to perform colony formation assay in cells depleted with Otub1. We hope the reviewer understands this point. We have also included time course studies for the expression levels of Otub1, p53, and p21 in cells transfected with scrambled or Otub1 siRNA followed by treatment with Eto. Our results confirmed that knockdown of Otub1 significantly attenuated p53 stabilization and activation in response to DNA damage (New Fig. 9C). Also, the levels of Otub1 were not significantly changed in response to Eto treatment (New Fig. 9C). We also observed that knockdown of Otub1 significantly attenuated the induction of p21 and mdm2 mRNAs by treatment with Eto (New Fig. 9H). These results further confirmed that Otub1 is critical for efficient p53 activation following DNA damage.

(2) "The cytoplasmic localization of Otub1 should be confirmed in other cell lines and especially in non-transformed cells; the p53-Otub1 binding, in a time course analysis, should be verified in the cytoplasmic compartment to confirm the model the authors suggest. Moreover, p53 unable to localize at the cytoplasm (by mutation of NES or leptomycin B treatment) should be used to confirm the model".
Our response: We have confirmed the cytoplasmic localization of Otub1 in several other cell lines such as RKO cells as well as in non-transformed WI38 cells (New Figs 10 C, 10D, S8B). We have shown the p53-Otub1 binding in a time course analysis in new supplementary Fig.S8C. We have generated a nuclear export signal (NES) mutated p53 (p53L348A/L350A), which is defective in nuclear export and thus retained in the nucleus (Stommel et al, 1999). We found that Otub1 does not significantly reduce MDM2-mediated ubiquitination of this mutant p53 (New Fig. S10E). Thus our data support that Otub1 suppresses p53 ubiquitination in the cytoplasm.

(3) "Finally, the results in figure 7 and 8 evidence a different activity of Otub1 following UVC in comparison to the other DNA damaging agents. Indeed, Otub1 seems ineffective in altering p53 levels upon UVC. This could be related to the dose of UVC used: 40J. This is probably a lethal dose that activates the apoptotic response (In fact, p21 levels decrease). This could discriminate Otub1 function following different cell responses rather than in different cell compartments. This could also solve the contradiction of Otub1 function in DNA damage response. As it has been suggested, Otub1 may set the threshold for initiating a transient DNA damage response (resulting in a growth arrest) (Nakada 2010). Conversely, its inactivation might switch on a permanent and fatal cell death response. The authors should verify and compare the functionality of Otub1 between these cell responses".
Our response: Thanks for the suggestions. We have performed dose-response for UV treatment as shown in new Fig. 9F. The results showed that knockdown of Otub1 markedly reduced the p53 induction to the similar extent in response to different doses of UVC treatment. The p21 protein is degraded by UV even in low dose, which is consistent with other reports (Bendjennat M, 2003 and Lee H, 2004).
I agree with the reviewer's notion that physiological function of Otub1 might be to set a threshold for RNF168 activity to maintain a proper levels chromatin ubiquitination. As mentioned above, it is very likely that the function of endogenous Otub1 is that upon DNA damage, it dissociates from the Ubc13/RNF168 complex while it targets UbcH5/MDM2 complex to activate p53, allowing cell cycle arrest and DNA repair. This may occur at transient or mild DNA damage condition. The same is true when cells receive lethal DNA damage signal, endogenous Otub1 may completely dissociate from Ubc13/RNF168 (inactivation of Otub1), resulting in sustain defect of DNA repair and sustained p53 activation and consequent cell death through apoptosis. In either case, our observed effect for Otub1 in the regulation of p53 signaling is consistent with its role in regulating chromatin ubiquitination. By the same token, Otub1 also plays a crucial role in setting a threshold for UbcH5 to maintain homeostatic levels of p53 under physiological conditions, via stoichiometric interplay with the MDM2-UbcH5 complex. Thus, transiently knockdown of Otub1 reduces p53 levels and likely facilitates DNA repair at specific conditions (such as defective ATM signaling) (Nakada et al, 2010), providing a survival factor for cells. Whether complete inactivation of Otub1 causes permanent cell death response associated with defective DNA damage repair as suggested in the Nakada study (likely in a p53-independent manner) would be beyond the scope of this single study. This topic is certainly our future direction and we appreciate very much this reviewer for the constructive suggestions and comments.

Specific points:

1. "Fig.1B: the blot of p21 is missing".
Our response: Thanks. The p21 blot has been added into the new Fig. 1B.

2. "Figure 2B: FACS analysis is not reliable as apoptotic assay and the signal of PARP cleavage is really weak. Authors should perform more specific assays (TUNEL, annexin, caspase-3 activation etc."
Our response: Thanks. The detection of Otub1-induced apoptosis by Annexin Vstaining have now been shown in the new Fig. 2B

3. "MDMX is also known as MDM4. For clarity, the authors should report the double nomenclature at the beginning".
Our response: Thanks. MDM4 was added as suggested.

4. "In Fig 4B, specificity of the binding between endogenous proteins should be confirmed by siRNA against p53 or Otub1".
Our response: We further confirmed the binding between endogenous Otub1 and p53 using control with Otub1 siRNA knockdown as shown in the new Fig 3B.

5. "The authors should report the cell line used in each panel of the figures. This would increase reading clarity".
Our response: Thanks. The cell lines have been added into most of the figures as

suggested.

2nd Editorial Decision 24 October 2011

I am sorry for the delay in getting back to you with comments on your revision, but in this case it unfortunately has taken a bit longer than anticipated to get the full set of comments back. I have now heard back from the three referees and as you can see below the referees appreciate the revisions made and find the paper suitable for publication here. The referees have a few issues that should be taken care of before publication here. I don't know if you have data on hand to address the first comment raised by referee #1 ("... important to show how the binding of Otub1 to p53-mdm2 complex changes with DNA Damage ..."). If no, then we can discuss this issue further. Once we get these last issues resolved then we will proceed with the acceptance of your paper in the EMBO Journal.

I look forward to seeing your final version

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

Overall, the authors did a good job in addressing the concerns, and have strengthened a model in which Otub1 stabilizes p53 through non-canonical mechanisms, namely inhibition of Mdm2-UbcH5 mediated ubiquitination of p53, rather than direct deubiquitination of p53. Residue D88 of Otub1 appears to be critical for mediating this effect, since its mutation to alanine abolishes E2 binding and the ability of Otub1 to suppress p53 polyubiquitination. However, the D88A mutant binds more strongly to p53, providing rationale for its behavior as a dominant negative inhibitor of endogenous Otub1 in cells.

From a functional point of view, I think it would be important to show how the binding of Otub1 to the p53-mdm2 complex changes with DNA damage, (i.e. increased binding of Otub1 to p53/mdm2) and to show that this correlates with p53 hypo-ubiquitination and stabilization.

On a small note, the recovery of unmodified p53 in Fig 4A and Fig 5C (Ni-NTA ubiquitination assay) is not adequately explained, but since the activity of Otub1 towards p53 is verified by other approaches (such as in Fig.4B and Fig.4C), this probably isn't a major concern.

Minor points:

p.21, line 7 - data further, not "further data"
p.22, line 1 - "poly-ubiquitination"
p.S3, line 16 - does, not "dose"
p.S4, line 11 - cell line should be H1299-tet-Otub1, not Otub2.

Referee #2:

The authors have adequately addressed the substantive concerns of the previous reviews. The manuscript is now acceptable for publication in EMBO J.

Referee #3:

The manuscript has been greatly improved and the authors have provided compelling evidence for the new proposed model.

I suggest some minor revisions in the text:
1. The following figures are not mentioned in the text: supplementary figure 1F and1G, S8C.
2. In figure Fig S5B, indicate with an arrow the monoubiquitinated p53 band (there are several bands).
3. Fig6B and C do not demonstrate the presence of a complex (this would require a triple immunoprecipitation). Please mitigate the sentence.
4. Page 15. "To understand whether Otub1 suppresses p53 ubiquitination by suppressing Ubc5, the Mdm2 cognate E2, in the presence of Mdm2." Please correct the sentence.

2nd Revision - authors' response 04 November 2011

Responses to reviewers' comments:

Response to Reviewer #1:

"From a functional point of view, I think it would be important to show how the binding of Otub1 to the p53-mdm2 complex changes with DNA damage, (i.e. increased binding of Otub1 to p53/mdm2) and to show that this correlates with p53 hypo-ubiquitination and stabilization."
Our response: This is indeed important, although it is difficult to examine the endogenous Otub1-p53-MDM2 complex (as mentioned by the reviewer #3 below). Also, it is well known that DNA damage attenuates MDM2-mediated p53 ubiquitination and degradation. However, we have now examined the interaction of Otub1 with MDM2 and found that Eto treatment increased the binding of Otub1 to MDM2 (as well p53) (new supplementary figure 6G), more significantly at the tested early time point (5 hours), suggesting that Otub1 targets MDM2 in response to DNA damage as well. This correlates with the increased p53 stabilization following Eto treatment.

Minor Points:

"p.21, line 7 - data further, not "further data"
Thanks, it has been corrected.

"p.22, line 1 - "poly-ubiquitination"
Thanks, it has been corrected.

"p.S3, line 16 - does, not "dose"
Thanks, it has been corrected.

"p.S4, line 11 - cell line should be H1299-tet-Otub1, not Otub2."
Thanks, it has been corrected.

Response to Reviewer #3:

1. "The following figures are not mentioned in the text: supplementary figure 1F and1G, S8C."
Thanks. These results have now been included in the revised text.

2. "In figure Fig S5B, indicate with an arrow the monoubiquitinated p53 band (there are several bands)."
Thanks. The monoubiquitinated p53 bands have been indicated in the revised figure S5B.

3. "Fig6B and C do not demonstrate the presence of a complex (this would require a triple immunoprecipitation). Please mitigate the sentence".
The reviewer was probably pointing to Fig. S6D. We have mitigated the sentence.

4. "Page 15. "To understand whether Otub1 suppresses p53 ubiquitination by suppressing Ubc5, the Mdm2 cognate E2, in the presence of Mdm2." Please correct the sentence".
Many thanks. The sentence has been corrected.

